CHEMICAL STUDIES OF MELANIN PIGMENT AND THEIR GENETIC INTERPRETATION

by

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INTRODUCTION

Differences in color of mammalian integuments, avian feathers, and other corresponding structures in other animals have been considered to be due to either (a) differences in quality and quantity of several pigments, more or less related (54, 56) or (b) the degree of presence or absence of a single pigment substance (46, 59).

If the alternative (a) is true, there remains the problem of determining the precise chemical and physical relationships between the various colors. The experimental work to be reported has a bearing here. If the alternative (b) is correct, only such factors as size and disposition of the pigment granules in the matrix, their quantity, the association with diffuse pigment, and the physical structure of the matrix need be considered in comparing different colors and determining the differences between them. The purpose of the present study is to review the literature on the subject of melanogenesis and to obtain some critical evidence whereby one of the two above hypotheses may become more firmly established. Guinea pig hair of different colors and intensities provided the pigments studied. A statistical method of comparing their spectrophotometric curves in alkaline solution will be proposed.

REVIEW OF LITERATURE

Definition of Melanin

Chemically Defined. The use of the term "melanin" has not been restricted chemically to any very definite group of substances--partly because the chemistry of these pigments has not been very successfully investigated. Hlasewits and Habermann (Riddle, 68) recognized in 1873 the necessity of oxidative processes in the formation of artificial "melanins". Kruksnberg observed in 1878 the production of dark brown to black pigments by certain organisms growing on media containing tyrosine, and referred to them as "melanin-producing" (Afanasiev, 5). The following list includes some of the chemical definitions of melanin used since that time:

(a) the colored products given by the enzyme, tyrosinase, acting upon tyrosine, or related chromogens (12, 42, 67);

(b) the colored products secured by action of dopaoxidase on dopa (14);

(c) colored compounds produced by the ultra-violet irradiation of aromatic amino acids (79, 80);

(d) polymsrized quinonoids (52);

(e) a pyrrole nuclei oxidation condensation product (6, 61);

(f) a condensation product of a compound having a phenolic base like tyrosine with a derivative possessing quinonic struc-

ture (53);

(g) the brown, gray, or black substance formed when a reaction to give such a substance takes place between any material and a strip of paper dipped in meal worm haemolymph (75);

(h) the product of the reaction between cystin and protamine (11, 69);

(i) 3, 4 dihydroxyphenylcysteine-- $(HO)_2\ C_6H_3\ CH_2C(SH)(NH_2)COOH$ (20);

(j) the black coloration produced by any material which reacts with H_2O, H_2O_2, and $FeCl_3$ on heating to give such a substance(4).

Definition (a) is the one most often found. Not all these definitions are mutually exclusive, chemically. The tyrosinase--tyrosine reaction, for example, has been shown to produce compounds having a pyrrole nucleus, and also dopa in intermediate stages in melanogenesis (7, 67). Quinones are formed in the same reaction, as will be seen later in connection with Figure 1. Definition (g) depends on the fact that the mealworm haemolymph contains tyrosinase, and the melanin produced would be analogous to that of (a). (c) produces compounds very similar to the melanins of (a), except that the oxidation has not been performed by an enzyme. The products defined by a, b, c, d, e, f, and g are probably chemically very nearly identical. The products of irradiation of aromatic amino acids have also been termed melanoidins (51), but this name should be reserved for the dark products of acid hydrolysis of albuminoids and other substances (Spiegel-Adolf, 79).

Biologically Defined. The usual biological definition of melanins refers them to the pigments characteristically found in the skin and its derivatives, the eye, and certain parts of the nervous system of mammals (19). Melanins from mammals have been studied in the case of negroes (1, 94), whites (60, 78), cattle (17, 26, 45), pigs (85), horses (62, 95), sheep (19), mice (21, 25, 80), rabbits (78), guinea pigs (44, 70), and many others. But the same name is given to pigments from pigeons (55), cephalopods (30), insects (22, 36, 40), and certain plants, particularly the pigments formed on bruising (31). With pigments from such widely varying types of organisms designated melanin, it is little wonder that chemical analyses and schemes for melanogenesis are often dissimilar.

Conclusions. From these considerations, melanins appear to be those pigments that occur regularly in mammalian integuments, and are presumably formed by the action of an enzyme or other biological catalyst on a chromogen which may be tyrosine or a related compound. The evidence that natural melanins may or may not result from such a reaction will be given below. Artificial melanins, shown to be similar chemically to these natural melanins, may also be termed melanin.

There is no a priori reason why the melanins from mammals should be the same from genus to genus or species to species. That they may be so is indicated by antigenic studies (2), and by the great similarity of the spectrophotometric curves of black melanin from horses (95), mice (21), the guinea pig, and man (see experimental results of the present study).

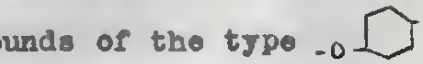
The Chemistry of Artificial Melanins

That the artificial melanins produced in vitro are essentially equivalent to natural melanins has been shown by the contributions of many earlier workers (Riddle, 68). Spectrophotometric evidence may be used to demonstrate the same thing (32, 80), although differences among melanins can be shown.

The Tyrosinase-Tyrosine Reaction in Vitro. The most important method of producing artificial melanins is that of the tyrosinase-tyrosine reaction. Although Bertrand's 1896 paper (12) is given credit by most reviews for the first mention of tyrosinase, Tauber (84) ascribed the original discovery to Bourquelot and Bertrand (18) in 1895. This monophenol oxidase was shown to be capable of oxidizing not only tyrosine, but any benzene ring with an hydroxyl group attached through a series of colored compounds to melanin (13). More recent work has demonstrated the necessity of the amino group (41) though the preparation of a "melanin" from benzene alone has been claimed (3). As has been mentioned, Hlasewitz and Habermann recognized that melanin formation was an oxidative process in 1873, and this fact was extended to the formation of melanin in the eye in 1899 (Riddle, 68). Tyrosinase was soon found in many animals and plants, but it has been recently denied that any satisfactory demonstration of its presence in mammals has ever been given (8), and that accordingly melanin formation in mammals cannot be related to tyrosinase. Methods which have been pro-

posed to avoid this difficulty will be discussed below.

Work performed largely by Raper and his co-workers has determined the course of the tyrosinase-tyrosine reaction in vitro (66, 67). The summary presented in Figure 1 is from Pryde (64).

Much work by Italian authors (6, 34, 72) on the pyrrole origin of melanin has been brought into this scheme by the demonstration that compounds of the type  may form pyrrole rings (7). Compound II is the "dopa" (3, 4 - dihydroxyphenylalanine) which will be mentioned in connection with the dopaoxidase theory.

The first colored substance is the reddish V. Between stages VI and VII, the color of the reaction mixture deepens from red through brown and violet brown to black. Haehn (42) considered these colors to depend on gradually increasing particle size, secured by some kind of polymerization or molecular aggregation. Since the enzyme is not required for this step, the only other possibility is that of auto-oxidation. Bertrand (13) showed that different substrates give differently colored intermediate and end products; however, differently colored natural melanins have never been related to such a difference in chromogen. They have been related to different stages of oxidation or polymerization, and this will be discussed under Theories of Mammalian Pigmentation.

The Dopaoxidase Theory. The discussion of this theory will be given mostly under Theories of Mammalian Pigmentation. However, certain objections to it provide the background for the dis-

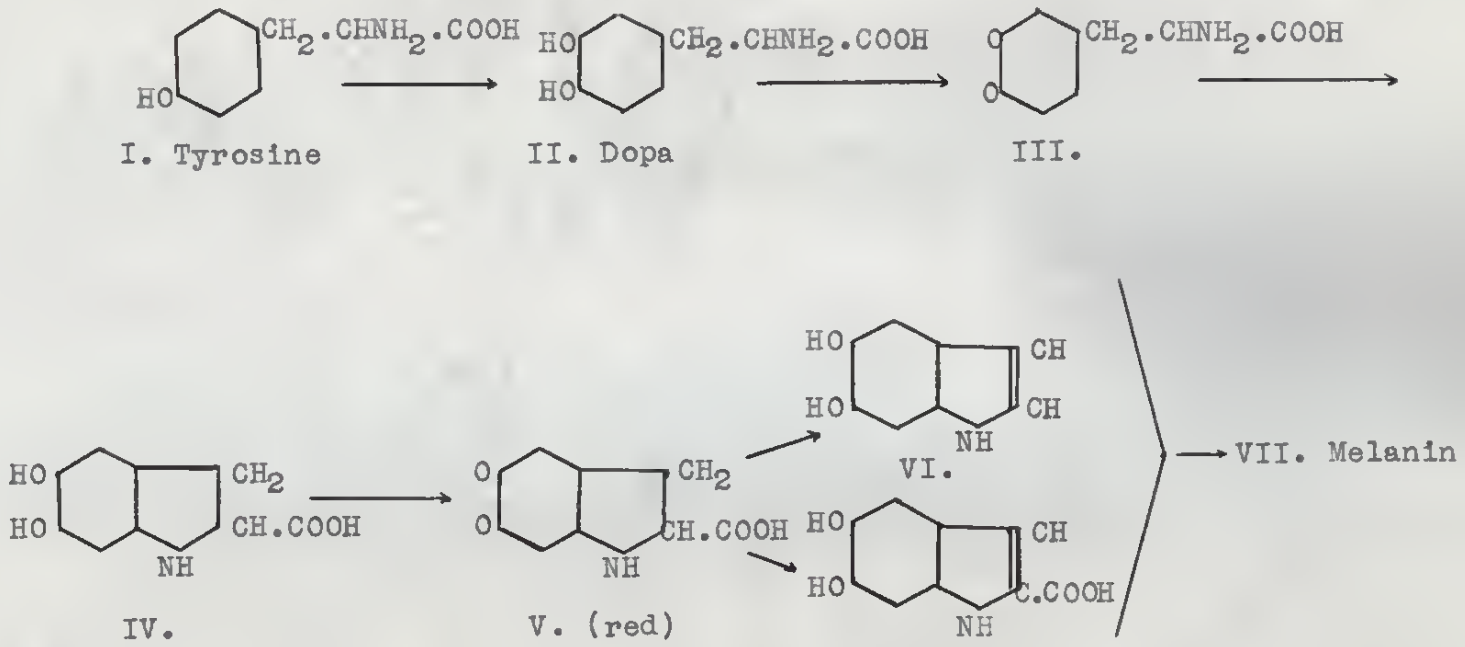


Figure 1. Action of tyrosinase on tyrosine. Optimum pH = 7.0 - 9.0. Substance V is the first colored compound of the reaction. Melanin forms from VI without the enzyme, more rapidly with higher pH, and probably by some kind of polymerization. After Pryde (64).

cussion of the cystin-protamine theory which is considered here. Briefly the dopaoxidase theory postulates that the natural melanins result from the action of a specific enzyme, dopaoxidase, on dopa or a very similar substance (16). That the reaction upon which this conclusion is based is enzymatic has been denied since the reaction proceeds without diminution in boiled sections of skin or after death (49, 69). In agreement with Tauber's classification (84) of biological catalysts, the agent responsible for the reaction could not be an enzyme, yet Tauber himself discussed dopaoxidase and cited no objections to its existence. Dopa is easily oxidized under many conditions with no enzyme present (63), and has been shown to react with biological catalysts not classed as enzymes (69). Because of certain results obtained by Ropshaw (69), explained below, the dopa reaction is probably not enzymatic.

The Cystin-Protamine Theory. A relatively new theory of melanogenesis has been elaborated by Ropshaw (69) and partially confirmed by Bellows (11). Since their experiments rest upon in vitro observations, they will be discussed here. Ropshaw found sulfhydrile--heat stable biological catalysts--present in the epithelial layer of the skin and investigated their relation to pigmentation. Protamines are compounds found conjugated with nucleic acid in the nucleus to form nucleoprotein. By extracting protamine from the nucleus in skins of albino and piebald rats, black, gray, and dominant and recessive white guinea pigs and rabbits, and allowing it to react with reduced glutathion in test tubes, white precipitates were secured which become black

in 2-3 days and returned to white in 2-3 weeks. No differences were found in the amounts of protamine or glutathion in the different skins, and the protamines from the different sources all gave the same reaction in vitro. Such a reversibly oxidizable system has been claimed for tyrosinase melanin (29) and natural squid melanin (30). Cysteine, which is formed from glutathion by cleavage, was tested similarly with the same result. In either case acidic pH's prevented the reaction, the optimum being 7.0-9.0. From the last experiment it was concluded that cysteine oxidizes in alkaline medium to cystin spontaneously and that this compound reacts with protamine. Similar tubes with cystin were then run and these gave the black precipitate in 4 days with some Liesegang rings. In 3-4 weeks the precipitate again became white through brown and reddish stages--giving some patterns resembling lepidopteran wings. Iron was present from the protamines. Melanogenesis according to Ropshaw, occurs, then, as follows: A stimulus, generally light, stimulates the intracellular enzymes, such as nuclease and nucleotidase, which cleave nucleoprotein (chromatin) into protamine and thymonucleic acid. The protamine moves by osmosis through the nuclear membrane and precipitates at the neutral point or above as colorless plastes. The alkalinity of the medium permits the autoxidation of cystaine to cystin and the reaction of this compound on protamine with Fe as a coferment in time forms melanin. The plastes are transformed to pigment granules. Post mortem pigmentation is explained by Ropshaw (69) as connected with the autolytic freeing of protamine, which is in accord with

Mierowsky's theory. No pigment is formed with O_2 because cysteine cannot yield cystin. Cystin and protamine are thermostabile and this explains the dopa reaction obtained in boiled skin sections, especially since dopa reacts with protamine. The increased dopa reaction obtained on illumination with ultraviolet light is explained by assuming that the light activates the enzymes which split off the protamine. Dopa plus buffered protamine solutions gave dark precipitates when the pH was above 7.0 and the intensity varied with the pH. Adrenalin and $AgNO_3$, both used as tests for melanin forming areas along with dopa, gave the same reaction with protamine under the same conditions. Bloch (15) and associates found the dopa reaction always negative in albino skins. Ropshaw explains this on the basis of his in vitro observations on pH in connection with the dopa--protamine reaction by saying that the cells of the pigment forming areas in albinos are below 5.58 in pH. Here the diffusing protamine cannot raise pH to neutral and does not precipitate. The sulfhydril is stable and does not oxidize in the acidic medium. Accordingly no pigment is formed. Ropshaw concluded that as far as protamines and sulfhydrils are concerned, the albinos, blacks, and whites are genetically alike--the problem concerns the variation in the third factor--pH. It has been previously reported (63, 69) that albino skins, white spots, and mammalian eyes are more acid in reaction than corresponding pigmented areas. It was concluded that weakening of the ensyme by the acid pH caused the resultant lack of pigmentation, but the difference in pH, if it exists, is just as subject to Ropshaw's

interpretation. It may be pointed out that the dopa technique includes buffering of the tissue exposed to dopa to about 7.34 and that the time before sectioning for observation may vary from 3 hours to 30 hours depending on the temperature (19, 71). Apparently the protamine or cysteine or both in the albino skin when changed to a pH of 7.34 do not reach the states in which they can react in this length of time, although those from the pigmented skin react even more quickly to give a black precipitate than in Ropshaw's in vitro experiments. Bellows (11) has offered confirmatory evidence of the cystin-protamine reaction as the source of melanin in the eye.

Although the objections to the dopaoxidase theory are well taken, some points in Ropshaw's theory are not clear. Protamine in the skin, it is claimed, is oxidized by some sulfhydril; yet protamine itself oxidizes dopa when the latter is applied to the skin--at least so Ropshaw explains the dopa reaction on the basis of the in vitro reaction between protamine and dopa. It would be more plausible that the sulfhydrils oxidized the dopa when it is applied to the skin, but apparently no in vitro confirmation of this reaction was secured. Also the natural melanins have been shown to give spectrophotometric curves which compare with those of dopa melanin and tyrosine melanin (31, 80). No spectrophotometric evidence to show that cystin-protamine "melanin" is the same as natural melanin has been adduced. pH differences in the skin of the same animal sufficient to account for the presence and absence of pigmentation, although reported, would seem to require thorough confirmation. If true, it might be

difficult to preserve the sharp delineation of albinos from colored animals, or of white spots on a pigmented background, since food and other environmental conditions might vary the pH. Sundstroem (83) has indeed observed albinos to become slightly pigmented in an artificially produced tropical climate, and Schultz-Allenstein (77) has produced marked melanin pigmentation in strips of skin with growing hair from albino rabbits when placed in moist oxygen at 30-36°. It would be interesting to check pH changes in the skin of animals fed diets deficient in the anti-gray hair factor (86). Recent work has correlated melanin content of hair and skin with the Cu content, and also shown that senile depigmentation is accompanied by decrease in Cu (74, 93). Attempts should be made to connect all these and other differences between white and pigmented areas of the same individual.

With the present evidence from spectrophotometric work, it is concluded that melanins as they occur in mammals are similar to those formed in vitro by the tyrosinase-tyrosine reaction. Until the cystin-protamine product is shown either to correspond chemically to the tyrosinase-tyrosine product, or is actually isolated from mammalian integuments, the above conclusion is certainly warranted. At the same time, Ropshaw should be given credit for recognizing the untenability of the dopaoxidase theory and for demonstrating the presence in the pigment forming areas of a heat stabile biological catalyst--the sulfhydrils.

Photosynthetic Melanins. A standardized method for preparation of melanins by ultra-violet irradiation of tyrosine,

tryptophane and phenylalanine solutions is given by Spiegel-Adolf (79). For a review of previous studies of photosynthetic melanins, her paper may be consulted. After purification by repeated centrifuging, her preparations were evaporated to dryness and dried. Consequently quantitative studies could be made without complicated corrections for impurities (25). The colloidality of solutions in alcohol and H_2O was indicated by strong Tyndall effects and the non-dialyzability of any coloring matter. H_2O_2 entirely bleached all three synthetic melanins, but no statement is made regarding color changes between the black melanin and the bleached end-products. For spectrophotometric evidence to be valid, it is required that the Lambert-Beers law hold true for the solutions studied. Some workers have thought that curves given by the colloidal melanin solutions might represent only scattering and not absorption (21), but Spiegel-Adolf (79) has shown experimentally that the three photosynthetic melanins obey the Lambert-Beers law. Hydrophobic colloids, in general, have been shown to obey this law (47, 79).

By plotting the spectrophotometric curves of the melanins obtained from tyrosine, tryptophan, and phenylalanine (log 1/cd log Io/I against wave length) straight lines, regularly descending toward the red end of the spectrum were obtained between 250 and 400 $m\mu$. Zwicky and Almasy's curves (95) for solutions of pigment from horse hair show some peaks in this same region, but the solutions contained dissolved hair protein which would account for them. That the photosynthetic curves are like those of natural melanin was demonstrated in a second paper by Spiegel-

Adolf (80) by comparison of mice melanoma melanin with photosynthetic tyrosine melanin. Small differences in the height of the tyrosine, tryptophan, and phenylalanine curves were found, even when placed on a comparative concentration basis, although the slopes of the curves were quite similar. These differences may provide the means of determining the substrate which actually forms mammalian melanin, if they can be related to the chemical nature of the substrate. However, Spiegel-Adolf considered the differences in the heights of the curves as more likely related to the degree of polymerization than to chemical differences.

Daniel's spectrophotometric results (21) with natural mice melanins extend observations on the straight line character of melanin curves plotted as above from 425 to 650 mμ and the experimental results to be reported confirm their general nature.

Miscellaneous. Definition (i) of melanin, p. 2, gives a relatively simple chemical formula for melanin based on elementary analyses of tumor melanin. No further mention of this compound as melanin could be found in the literature.

The number of substances which form "melanin" with H_2O, H_2O_2, and $FeCl_3$ (definition (j) p. 2) is so great as to cause the term to lose any particular significance. They include, according to Adler and Wiechowski (4): $PhNH_2$ and homologues, $PhNHNH_2$ and homologues and substitution products, phenols, quinones, aromatic mono-aldehydes and ketones, mono-COOH acids and phenol mono-COOH acids. Most of the 382 compounds tested gave the reaction. Aliphatic compounds did not, while terpenes,

Ph Ch-, C_5H_5 N-, $C_{14}H_{10}$-, and phenanthrene groups gave little or no reaction.

Theories of Mammalian Pigmentation

The Tyrosinase-Tyrosine Reaction. After the discovery of tyrosinase in a fungus by Bourquelot and Bertrand (18) it was soon demonstrated in many animals and plants. The following examples may be given: adrenal glands (50), meal worm, Bombyx, and other insects (22, 36, 68) in Sepia (35), in Loligo (68), in Halla (57), in horse tumor (37), and in the skins of rabbits and guinea pigs (23, 59, 65). Gessard (37) also discovered free tyrosine in the horse tumor and Dewitz (22) demonstrated the role of tyrosinase in the development of the natural pigment of an insect. Accordingly it seemed likely that v. Furth and Schneider's suggestion (87) that melanin pigmentation in the animal kingdom is the result of the action of tyrosinase on tyrosine would prove correct. However in 1937 Arnow (8) stated: "There is as yet, however, no direct proof that this mechanism operates in mammals, since tyrosinase has been isolated only from plants and the lower forms of animal life." The demonstrations of tyrosinase in mammals (23, 37, 59, 65) are apparently regarded as unsatisfactory.

This first theory of mammalian pigmentation was strengthened by the correlation of the naturally occurring colors--light red, red, chocolate, and black--with those observed in the different stages of the tyrosine-tyrosinase reaction (68, 89). The isolation of white "melanin" by Spiegler was interpreted by some

authors (81) as providing the final chromatic stage in the oxidation of tyrosine. Dominant whites were considered to have the power of oxidizing tyrosine to this final state, whereas albinos lacked either the enzyme or the chromogen and thus could form no melanin at all. Gortner (38) denied that white "melanin" was a melanin at all and considered it a keratin decomposition product determined by the method of extraction. He found several substances capable in inhibiting the tyrosinase-tyrosine reaction and proposed that dominant whites are characterized by such an inhibitor (39). If the intermediate stages in the tyrosinase-tyrosine reaction correspond to the actual pigments, it should be possible to demonstrate it in some way besides analogy. The only established case shown to account for natural pigmentation is not in mammals, but in Halla (33, 57). Accordingly the concept that the yellow, red, and brown mammalian pigments are incompletely oxidized stages in the production of black melanin has only a hypothetical basis, especially in view of certain evidence to be discussed under the relation of red and black melanin.

The Dopaoxidase Theory. In order to avoid the difficulty of demonstrating tyrosinase in higher animals, the dopaoxidase theory has been developed since 1917 by Bloch and his followers (14, 16). It may be briefly stated as follows: Pigment forming tissue contains an oxidase capable of converting l-dopa into melanin. This reaction is positive for the melanoblasts of higher vertebrates. There is a time, place, and intensity parallelism between the reaction and the natural formation of

pigment. The oxidase responsible is quite distinct from tyrosinase and reacts with l-dopa. It is concluded to be the natural pigment producing enzyme, dopaoxidase, with its substrate being l-dopa or a closely related compound.

Some objections to this theory have been mentioned in connection with the cystin-protamine theory above. If the agent that reacts with dopa within the cell is an enzyme, it is peculiar in its thermostability. Tyrosinase is inactivated by heating to 70°C. for one minute in the case of the potato beetle elytra (40). DuShane (24) has shown that the dopa reaction is not correlated at all with pigment producing areas in Amphibia, thus confirming Schmidt (24). Not only epidermal cells, but red blood cells, intestinal cells, occasional yolk cells and mucous skin gland cells were dopa positive.

Besides the dopa reaction, a number of other pigment forming reactions have been used to indicate natural pigment forming areas. Moncorps (58) has concluded that reactions given by fresh skin sections + $FeCl_3$ + p-hydroxyphenylpyruric acid, homoprotocatechuic, or dihydroxyphenylpyruric acids are fermentative processes, and that therefore specific enzymes play a part in pigmentation. The reaction was not given by heated skin. Saccardi (73) detected a localised chromogen, thought to be pyrrolic in nature, by using phenylazoxycarbonamide on skin sections. $K_2S_2O_8$ produces colored substances in basal skin epithelium and in other places, but the other reactants were not known to Schmidtmann (76).

While any of these reactions may provide the key to the

solution of the problem of melanogenesis, it is doubtful if any thus far described reproduce the actual reactions responsible. The dopaoxidase theory is in little better standing than the tyrosinase theory, while the cystin-protamine theory does not yet have any evidence that natural melanins and the cystin-protamine product are chemically similar. The cystin-protamine theory has given us a possible basis for the understanding of the dopa reaction and these other reactions in the skin, but the relation of the end product of pigmentation--melanin--to the in vivo reaction responsible for its production has not yet been found.

The Relation of Red and Black Melanin. Earlier workers (68, 89) inferred from the tyrosinase reaction that black melanin was the oxidized product of red melanin. Recent evidence indicates that the reverse is more likely to be true. Black melanin treated with H_2O_2 has often been observed to give red and yellow products (19, 52) although complete bleaching is obtained with longer treatment (79, 82). Arnow (9) compared the spectrophotometric curves of an alkaline extract of red human hair and a reddish solution of artificially oxidized dopa melanin and found them alike. He concluded that red melanin is an oxidized form of black melanin. A serious objection to this conclusion is Zwicky and Almasy's statement (95) that red and black melanins (from horses) cannot be spectrophotometrically distinguished. Obviously, if this is true, Arnow's comparison of red melanin and the red oxidized product from black melanin would be meaningless. Arnow objects to Zwicky and Almasy's conclusion on the basis that their published curves for red and

black do not appear to him similar, and this questioning of the validity of Zwicky and Almasy's conclusion provided the starting point for the present investigations. The relationship between red and black melanins is accordingly an important question and will be again taken up in the discussion of experimental results.

Quantitative vs. Qualitative Differentiation of Differently Colored Pigments. The two theories of mammalian pigmentation thus far discussed have suggested that differences in color depend upon the stage of oxidation or polymerization of substances corresponding to those found in the in vitro tyrosinase-tyrosine reaction. That there are such qualitative differences in pigment is supported by a number of investigators. Lodemann (56) believed red and black are of a similar chemical nature, but at different stages of oxidation. Lloyd-Jones (55) found this evidence that pigmentation may be qualitative: (1) red granules were usually smaller than black, but one variety of red showed the largest granules, and (2) red feathers contained more pigment than black. The qualitative concept of pigmentation differences is usually taken to require that red pigmented structures contain a lesser amount of the same material present in larger quantities in black pigmented structures. Harman and Alsop (43) and Harman and Case (44) described in embryological studies of the guinea pig the presence of black, red, and chocolate granules, a diffuse reddish pigment, and also colorless granules. No difference in the time of development of the different colors was noted (44) although Esskuchen (27) observed

that reddish brown pigmentation appeared in cattle embryos three and a half months later than black pigmentation. In Little's view (54) three distinct pigments exist in mice--yellow, brown, and black. Serra (78) has used chromatographic adsorption methods to separate two different melanins from rabbit hair and three from human hair.

Certain authors have claimed that pigmentation differences in mammals have only a quantitative basis. Fasal (28) found dark hair to contain thirty times as much pigment as light hair and concluded that the difference in color depended chiefly on the amount of pigment. Jankowsky (46), by immersing colorless hair in solutions of $AgNO_3$ for varying lengths of time reproduced all shades of hair color and decided that macroscopic hair color is determined by varying amounts of the same pigment. Onslow (59) found that the same melanin gave all shades from yellow to black and that accordingly pigmentation must be quantitative. Boyd (19) showed that the dopa may react in vitro to give all shades of color and confirmed Haugg (19) that color tones are quantitative, not qualitative, and due to the arrangement and distribution of pigment in the hair follicle. It is hardly reasonable, however, to thus regard the differently colored products of dopa oxidation, depending on the stage of oxidation as quantitative differences of a single pigment substance. If one treats black melanin with H_2O_2 a yellow product is secured which is thought to correspond to yellow melanin naturally occurring. But it cannot be admitted that the black melanin and its yellow oxidation product are the same compound, quantitative

differences in which have produced the difference in color, because the two have not been differentiated quantitatively, but only chemically or qualitatively. The evidence of Zwicky and Almasy already referred to that red and black melanins are qualitatively identical is more serious, and the specific purpose of this investigation was to determine whether Zwicky and Almasy's conclusion holds true for guinea pig melanins (See Experimental Study of Guinea Pig Melanins).

Conclusions. The conclusion must be that the manner in which the different melanins are formed in vivo is not known. Though the end product can be assumed to be closely the same as that formed by tyrosinase acting on tyrosine or related compounds, or even by the oxidation of dopa in the presence of O_2, the paths by which that end is reached in the organism have not been elucidated. Much research with the melanins is yet possible. Spectrophotometric observations of Ropshaw's cystin-protamine product should be obtained. Comparison of physical and chemical properties of melanins from many different sources has never been performed under standardized conditions. The study of spectrophotometric differences between pure synthetic products in relation to the particular substance used as a substrate or chromogen should be carried out in order to determine the actual melanogen in mammalian pigmentation. Methods for separation of melanin from kerstin are not satisfactory and new solvents can perhaps be found. The relation of Cu, pH, and other conditions to melanin production in vivo should be investigated and correlated. How the agouti pattern, common in wild rodents, arises

remains to be determined. The application of physical chemistry methods to these and other problems should provide us in the future with much of the knowledge needed to understand the manner of production and disposition of melanins in the hair and skin.

EXPERIMENTAL STUDY OF GUINEA PIG MELANINS

Introduction

Spectrophotometric studies of melanin have been mentioned in the preceding sections (9, 21, 31, 32, 79, 80, 95). The special significance of Zwicky and Almasy's conclusions in determining the correctness of one or the other of the two theories regarding color differences has been indicated. Their statement that red and black melanins cannot be distinguished spectrophotometrically would almost establish the theory that pigment differences are of a quantitative nature. However, if the two curves were given by the same chemical substance, it should be possible to make them coincide by multiplying one or the other by some concentration factor. Examination of the data showed that this could not be done, and since Arnow (9) had previously expressed the opinion that Zwicky and Almasy's red and black melanin curves were dissimilar, the present work was undertaken to obtain more evidence.

Methods

Solutions for spectrophotometric comparison were made up in the following manner. After defatting by extraction with CCl_4

for two or more hours in a Soxhlet apparatus, the hair (0.125 gm/sample) was boiled in 1 per cent NaOH for approximately two hours. Water was added as needed to keep the volume approximately constant. After cooling, the solutions were filtered, made to 100 cc volume, and a portion used in a Bausch and Lomb spectrophotometer to determine the absorption curve. The setting of the spectrophotometer was checked to the Sodium D line during the course of the experiments. Absorption was determined at four wave lengths, since it was found that these sufficiently described the curves between 470mμ and 600mμ. The averages of the readings, usually ten in number at each point and often made by two individuals independently, were used to determine the curve.

During filtering some pigment was usually retained on the filter paper. The amount so held apparently depended on variations in heating, the amount of absorption by the keratin residue, and the kind of pigment involved. Black melanin solutions left the filter paper quite black, while red melanin was only slightly retained under similar conditions. Daniel (21) has noted that the shape of the curves is not affected by this filtering out of part of the pigment, since all the pigment of a given sample when dissolved gave the same curve as the first fraction. The curves given by solutions of black melanin from guinea pig hair, however, were altered by five hours boiling time; therefore, the period of extraction with boiling alkali was limited to two hours or less.

The description by which a particular melanin is designated

is presented in column three, Table 1 and in column four, the known genetic composition. The first two are both black, but

Table 1. Description and genetic composition of melanins.

Number	Guinea pig number	Description	Genetic composition
1	W908.4	intense black E	P Sm Ee C aaBb didi
2	W23.1	intense black e^p	P Sm e^p C aaBB didi
3	V200.1	c^r intense black	P Sm e^p c^rc^r aaB
4	V876.4	c^r dilute black	P Sm e^pe c^rc^r aaBB
5	X91.1	intense chocolate	P Smsm E-C-aabb didi
6	S756.2	cherry red bb	P Sm eeC bb
7	U218.4	cherry red B	P Sm eeC Bb
8	W311.1	albino	c^ac^a
9	--	dopa-melanin	--
10	--	intense black human hair (Caucasian)	--

they differ in that the second comes from an animal also having red spots (due to $\underline{e^p}$) and the first from one that is entirely black (due to $\underline{E}$). One would expect the two black melanins to behave similarly when compared spectrophotometrically. Most black and chocolate guinea pigs have hair less intensely pigmented at the base than distally; however the $\underline{di}$ gene carried by melanins one, two, and five causes the hair to be almost uniformly pigmented. Melanins three and four differ from one and two in carrying gene $\underline{c^r}$ in place of $\underline{C}$, and for this reason such

hair appears less intensely black. Of the two, number four is definitely more dilute in appearance than three, although the inheritance of the genes responsible for this difference has not as yet been determined. The black guinea pig melanins might be ranged, then in order of decreasing intensity of the blackness of the hair: 1 and 2<3<4. Melanin five corresponds to one and two in intensity, but since it carries gene b for chocolate instead of B for black, is intense chocolate in color. Numbers six and seven also differ only in that one carries b, the other B, but since these animals are also ee, which produces red, the chocolate pigment in number six and the black pigment in number seven are restricted to the skin of the ears, eye lids, nose, etc, leaving the hair entirely red. Cherry red is a term which distinguishes the dark red characterizing these animals and others of "show" type from the slightly lighter red ordinarily found in laboratory stock. The albino, number eight, probably contains no melanin and was included in order to have a check on the effect of keratin degradation products. Dopa-melanin, number nine, is the designation given the solution resulting when 1 gm dopa (3, 4-dihydroxyphenylalanine) in one liter of H_2O stood for approximately a year stoppered and in a paper cover, during which time it had oxidized to a very dense black solution with the formation of a slight precipitate. The tenth melanin was obtained by dissolving samples of very intense black Caucasian human hair.

Results and Discussion

Both Daniel (21) and Spiegel-Adolf (79) have plotted the logarithm of the optical density (optical density = log Io/I) against wave length for their spectrophotometric curves of melanin solutions. While there is no theoretical justification for this procedure, it affects each curve similarly and introduces no error. In so doing, a nearly straight line results. This method of graphing was adopted in the present experiments, but in addition, statistical methods of comparing the curves were used in order to obtain a more accurate measure of slight curve differences. A straight line may be fitted to the experimental points by the method of least squares, and this method gives a value, the regression coefficient, which expresses mathematically the slope of the calculated best line. For each sample of melanin, the logarithms of the optical density at the four wave lengths were used and the regression coefficient calculated. The average regression coefficient and the number of samples for each melanin are given in Table 2.

Curves calculated by the above method have been plotted in Plate I for representative samples of several of the melanins. In order to compare these and Daniel's data, her curve for heterozygous chinchilla mouse melanin is also given. Genetically, this melanin corresponds to $\underline{c^r}$ black guinea pig melanin and would be expected to have a similar spectrophotometric

Teble 2. Average regression coefficients of log optical density against wave length for alkaline solutions of various melanins.

Number	Description	Solutions prepared by 2 hours boiling		Solutions prepared by 2 weeks extraction of hair at room temperature	
		Number of samples	Regression coefficient	Number of samples	Regression coefficient
1	intense black E	10	-.020	1	-.042
2	intense black e^p	3	-.020	1	-.040
3	c^r intense black	6	-.025	1	-.040
4	c^r dilute black	2	-.033	1	-.040
5	intense chocolate	2	-.029		
6	cherry red bb	6	-.060	1	-.069
7	cherry red B	2	-.068		
8	albino	4	-.047		
9	dopa melanin	3	-.019		
10	intense black human hair	2	-.016		

curve. Regression equations for the curves of Plate I are as follows:

intense black $\underline{E}$	E = 1.018-.019X
$\underline{c^r}$ intense black	E = 1.175-.025X
cherry red $\underline{bb}$	E = 2.734-.059X
albino	E = 1.284-.048X

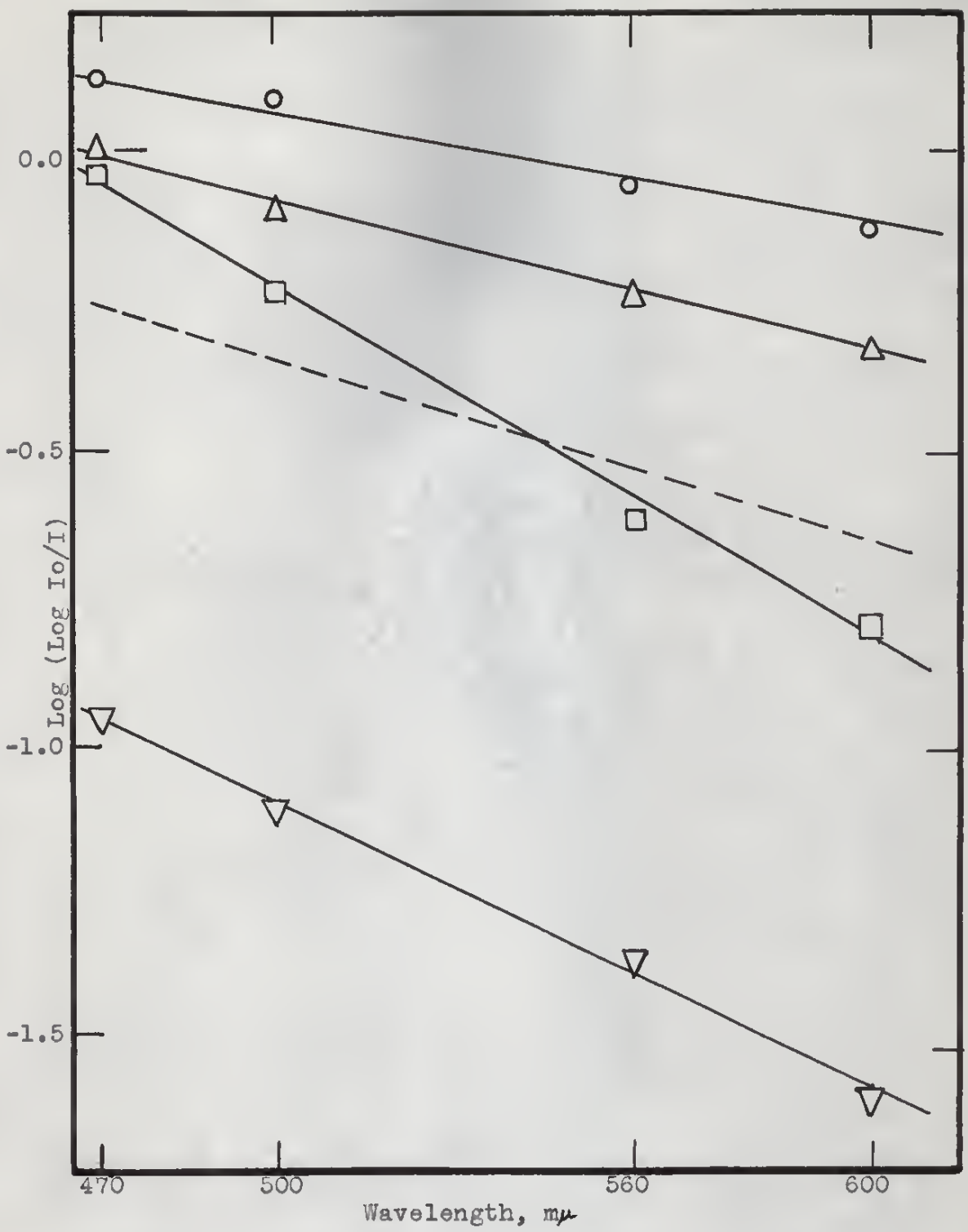
The regression coefficient is the number preceding the X and has been calculated as the change in logarithm of the optical density per change of 10mμ in wave length. The negative sign indicates the inverse relationship. The curves appear linear in the range investigated, and there is reason to believe that this relationship holds for melanins free from protein in the ultra violet (79).

The two black guinea pig melanin curves, $\underline{C}$ and $\underline{c^r}$, and the black (chinchilla) mouse melanin curve appear to have approximately the same slope in Plate I. Since that of the latter is about -.030, two curves whose regression coefficients differ by as much as .011 are not readily observed to be different in slope by inspection of such a figure. Such a degree of similarity between two curves has been taken by previous workers to indicate the melanins involved are spectroscopically indistinguishable and probably chemically alike. Within the above limits, it is evident from Plate I that black guinea pig melanins give curves like black melanins from mouse hair and from horse hair (since Daniel's black mouse melanin curves are very similar to Zwicky and Almasy's black horse hair curve). However, as will

EXPLANATION OF PLATE I

Spectrophotometric curves of alkaline solutions of guinea pig hair melanins plotted log (log Io/I) against wave length:
○ = $\underline{C}$ black; △ = $\underline{c^r}$ black; □ = cherry red $\underline{bb}$; ▽ = albino.
---- = heterozygous chinchilla mouse melanin (after Daniel, 21).

PLATE I



be shown later, the slight difference in slope between these two black guinea pig curves --- .005 in amount --- is statistically significant. It follows that a statistical treatment is more likely than visual inspection to reflect the differences which do occur.

The curve for red guinea pig melanin, however, may readily be distinguished statistically from those of any of the black melanins. It is evident even from inspection that no shifting of the red curve up and down by use of concentration factors (which does not change the slope of the curve) can make it coincide with any of the black curves. Zwicky and Almasy's statement that black and red melanins may not be distinguished spectroscopically does not hold for guinea pig melanins.

This evidence also would not support an explanation that the fundamental difference between hairs of different colors depends entirely on the amount of pigment present. Russell's results (70) seem to lead to the same conclusion, for she found that intense red guinea pig hair required more $KMnO_4$ to oxidise the pigment than did an equal weight of the extremely dilute black and chocolate hairs. Einsele (25) has suggested that qualitative differences may exist even between black and chocolate melanins for equal weights of pigment from mice of different genotypes dissolved at the same concentration gave different color intensities in a colormeter. In the present experiments, the regression coefficient for intense chocolate guinea pig melanin

falls between those of the two c^r black melanins. Consequently some physical difference not reflected by the spectrophotometric method must differentiate chocolate from black melanin.

The other curve in Plate I, albino, has been used by Zwicky and Almasy (95) as a correction for the absorption of keratin degradation products. They subtracted this correction from the pigment curves. This procedure has not been followed because the results are only slightly changed and the albino curve has been found to be subject to considerable experimental error.

The extreme range between regression coefficients for the same melanin was .013 and occurred between two samples of cherry red bb. Since the other five samples were grouped around -.062, it is not unlikely that the low sample (-.050) was in error; nevertheless all six are averaged together to give the regression coefficient -.060 for cherry red bb melanin. The ten samples of intense black B melanin ranged from -.017 to -.024, the next largest spread in values for a given melanin. These averaged to -.020 and the variation can be attributed to experimental error. When the regression coefficients for c^r intense black and cherry red bb were compared statistically, it was found that the probability of their being random measurements of the same population regression coefficient is far less than one in a thousand ($t = 25$, 1% level = 2.7). Statistically highly significant differences may also be found among the regression coefficients of the black melanins (for intense black E and c^r intense black, $t = 9$, 1% level = 2.7). This may confirm Einsele's evidence for qualitative differences among different

black and chocolate melanins, but the number of samples is small. The $\underline{c^r}$ dilute black curve is significantly different from the intense black $\underline{E}$ curve, and moreover bears the same relation to it that the most dilute black curve of Daniel bears to her intense black mouse melanin curve. As Daniel stated, the differences between the two appear small when the plotted curves are examined. However, the use of regression coefficients may indicate whether such small differences are consistent or without significance. Daniel has further suggested that the curves may represent scattering and not true absorption. If this is true, further study of melanin curves might lead to information of the relative particle sizes involved. Even if melanin solutions are not true solutions, conclusions from spectrophotometric observations are probably valid, since the Lambert-Beers Law has been shown to be generally true for hydrophobic colloids (47), and in particular for solutions of melanins (21, 79, 95).

Zwicky and Almasy state that their solutions obtained by extraction of the hair for two weeks at room temperature did not alter in optical properties on long standing. Others found, however, that solutions prepared by boiling were apparently bleached by exposure to light or ultra violet light or on further boiling (21, 25). Daniel has presented curves from samples of hair boiled 30 minutes and 165 minutes to show that the slope is unchanged although the apparent concentration decreases. In the present experiments not only were changes found in the apparent concentration of pigment as measured by the height of the spectrophotometric curve, but also the slope of the curve

was altered on standing.

Table 3 illustrates these changes for intense black E melanin. The first line records the average optical density of two samples at 470mμ at the time intervals indicated, while the second line gives the average regression coefficient for the two curves. The values at 120 days are from a different sample of the same melanin. Red melanin solutions in general changed much less rapidly but must be further studied before arriving at definite conclusions. The average regression coefficient for two samples of red melanin changed from -.062 to -.069 after 18 days.

Using Zwicky and Almasy's method of extracting the hair two weeks with alkali, solutions were prepared which gave the regression coefficients in the last column of Table 2. These values check quite well with those of Table 3 for between 11 and 18 days; so it may be concluded that the changes which take place in the boiled solutions on standing also occur when Zwicky and Almasy's method is employed. The greater similarity of the curves after 14 days may explain in part why Zwicky and Almasy decided the curves for red and black melanin are alike.

The change in the slope of the curve of the black melanin solution on standing obviously brings it nearer that of the red melanin curve. Arnow's oxidation (9) of a solution of dopa melanin in air to a reddish solution having the same spectrophotometric curve as an extract of red human hair has previously been mentioned. He concluded that red melanin is an oxidation product of black melanin. A comparison of Arnow's red melanin

Table 3. Changes in optical density and regression coefficient of intense black E melanin solutions with time in 1 per cent NaOH.

Days	0	1	2	4	7	11	18	120*
Optical density at 470 mμ	1.53	1.23	1.18	1.02	0.91	0.82	0.74	0.23
Regression coefficient	-.022	-.025	-.028	-.032	-.033	-.036	-.039	-.048

*Readings at 120 days made on a different sample of the same melanin.

ourves with those of red guinea pig melanin shows that the latter are definitely more steep. The former would have a regression coefficient of approximately -.04 while that of red guinea pig melanin is -.06. A sample of dopa-melanin oxidized in this laboratory by bubbling air through it for two days in 1 per cent NaOH changed its regression coefficient from -.021 to -.039, but seemed to reach an equilibrium at that point. Treatment of this product with H_2O_2 produced a yellow solution with a regression coefficient of -.062. Arnow's conclusion that red melanin is an oxidation product of black melanin apparently holds for guinea pig melanins although red guinea pig melanin seems to have a definitely steeper curve than any other red melanin yet reported.

Some results of Bogart and Ibsen (17) and Ibsen and Bogart (45) may be re-examined in the light of the present results. In microscopic studies of hair of cattle and guinea pigs, they often found the medulla to be entirely black, even in white hairs (See Plate VI of Bogart and Ibsen, 17). They believed this to be black pigment because the medulla of such hairs became colorless on bleaching. When they observed red pigment replacing black in the cortex of black hairs after bleaching with H_2O_2, they interpreted this result as indicating that red was present but could not show until the black had been bleached to colorless. They state "We have shown that when black (B) hair is bleached, the red pigment becomes visible. Some might suppose that the red is only one of the stages encountered in the process of bleaching the black. That this is not the case is demonstrated by the fact that when the black pigment in white

hairs (which never contain red) is bleached, no red coloration is at any time apparent."

Spectrophotometric attempts to demonstrate black pigment corresponding to that in the medulla of Bogart and Ibsen's white cattle hairs failed. White Ayrshire hair extracted according to the procedure of these experiments gave a curve similar to that of albino guinea pig hair. Bogart and Ibsen found albino cattle hair to have these same black medullas, but that such an amount of black pigment would not color the solution and give an appreciable curve is inconceivable. The disappearance of the black medulla on bleaching must have been due to some clearing action of the H_2O_2, and the non-appearance of any red oxidation product of black pigment to the fact that no black pigment was present. The red pigment appearing in black hairs, on bleaching was H_2O_2 is best explained as an oxidation product of black melanin.

Conclusions

1. In contrast to Zwicky and Almasy's results with horse melanins, red and black guinea pig melanins may be easily distinguished by the spectrophotometric technique. These results confirm the theory of mammalian pigmentation that differences in hair color are both qualitative and quantitative.

2. Confirmation of Arnow's statement that red melanin is an oxidation product of black melanin has been secured by observing the changes that take place in solutions of black melanin on standing and correlating these with the changes pro-

duced by oxidizing black dopa-melanin.

3. The use of regression coefficients for log optical density plotted against wave length is proposed as a means of comparing the absorption curves of melanin pigments.

4. Some evidence that qualitative differences may exist between different black and chocolate guinea pig melanins has been found. However, their curves are very similar to those of black melanins from mice, horses, and man, and also to that of dopa-melanin.

5. The regression coefficient for intense chocolate melanin falls within the range of those for different dilute black melanins; hence it is concluded that chocolate and black melanins are chemically alike and that the B locus probably determines some difference in physical state of the pigment present.

THE GENETIC INTERPRETATION OF EXPERIMENTAL RESULTS

A comprehensive theory of the processes involved in the melanin pigmentation of mammals has been proposed by Wright (89, 92) and related to the known color genes in guinea pigs. Figure 2 and the following discussion are taken from the latter publication (92).

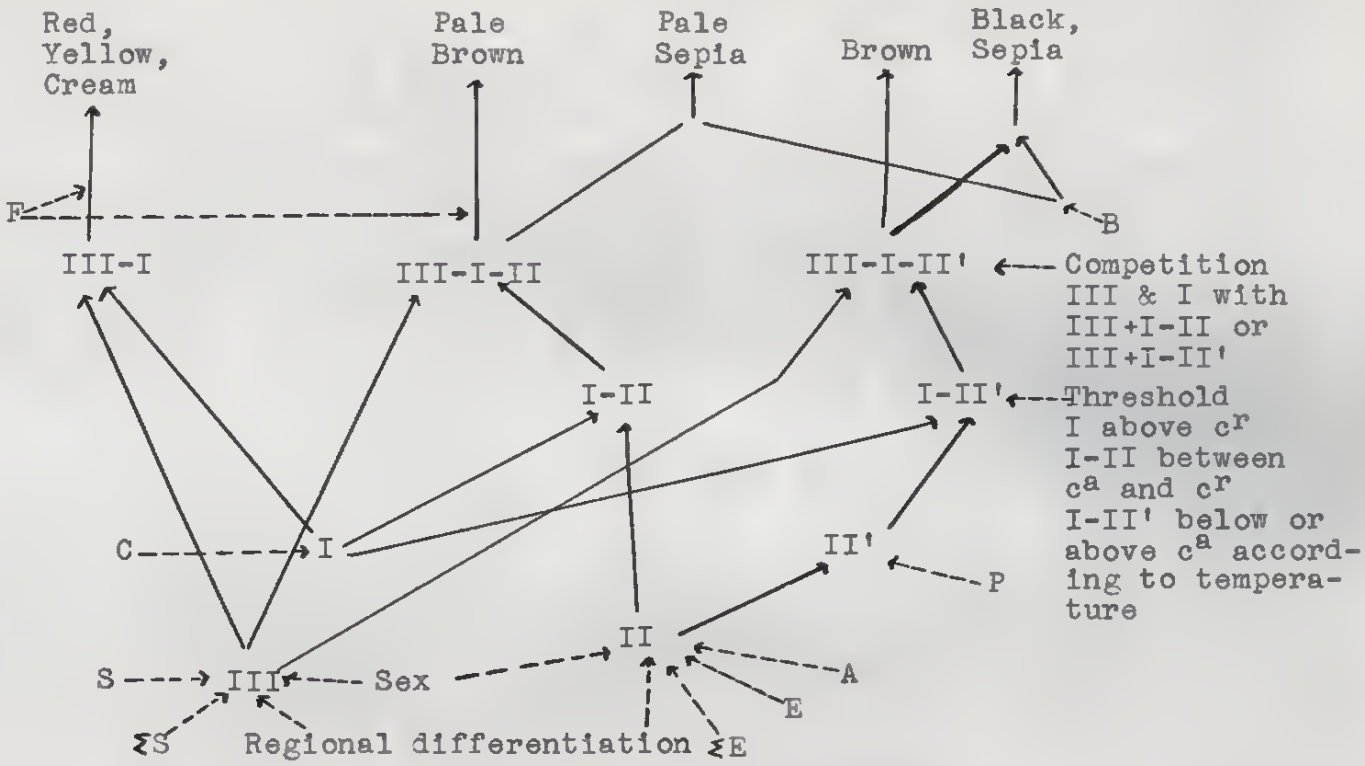


Figure 2. (Wright's Figure 10, p. 564 (92) Hypothetical processes involved in melanin pigment production and points of action of factors as deduced from the effects of factor combinations.

(p. 563-5) The deductions as to processes involved in color production and the points at which the known color factors have their primary effects are represented graphically in Figure 10 (See Figure 2 above). The albino series is represented as determining the quantity of substance I ..... This substance may unite with a substance labeled II..... The presence of II in a particular region is represented as determined by a number of conditions, including factors of the extension series (E), modifiers (ΣE), sex, regional differentiation, and factors of the agouti series (A). Onslow's discovery of an inhibitor of the action of tyrosinase in the skin of agouti rabbits suggests the mode of action of the A-series. The nature of substance II is represented as affected by the P-series. The action of the P-series must be located at this point on the strength of the evidence that it precedes the threshold and competition effects.

The union of substances I and II is represented as essential to the production of sepia (black) or brown pigmentation, while substance I without II is represented as essential for production of yellow. The evidence indicates that I and its products with II are ineffective below certain levels of production (of I), which vary with presence and nature of II as described .....

The two substances I and I-II are represented as competing (in the regions in which II is present at all) for union with a third substance (III). It is necessary to suppose that I-II is about equally effective whether modified by P or p, except for the threshold difference. The assignment of the action of the piebald series (S_s) and accessory modifiers (genes ΣS), sex, age of dam, etc., to substance III is arbitrary. It is merely known that something essential to all pigmentation is affected in an all-or-none fashion in different parts of the skin. This may be back of I instead of III.

The brown series (B, b) appears to act upon the precursors of sepia exclusively but regardless of modifications by P or p and without influence on threshold or competition with yellow. This effect is conveniently represented in the diagram as following these processes, although this is not a necessary conclusion.

Two distinct effects must apparently be assigned the F series. The threshold of yellow production is raised by f, but in this case without influence upon competition ..... Factor F is also an essential for any production of sepias in the absence of factor P.....

For the evidence upon which these and other interpretations included in Wright's hypothesis are based, see his papers (88, 89, 90, 91, 92). Considerable investigation will be required to determine the precise effect of each gene in definite physico-chemical terms, but some conclusions may be drawn from the present studies and review.

The C series of multiple allelomorphs (C, c^k, c^d, c^r, c^a) is apparently thought of by Wright (89, 92) in terms of the quantitative production of an enzyme. Whether an enzyme is concerned in production of melanin in mammals is not definitely known, and other factors such as pH have been related to the C-series (65, 69). More study of this question is required before the actual role of the C-series can be established. Substance II in Wright's scheme is produced by gene E (and affected by other genes) and determines the presence of black or chocolate pigment when Substance I is present above the necessary threshold (between c^a - c^r). Since this genetic difference between E and ee must be related in some way to the chemical difference between the black and red pigments resulting, and since this chemical difference has been shown to be one of oxidation, there are these possibilities:

(1) E determines the presence of a less efficient oxidizer than ee, or

(2) E produces an inhibitor of oxidation past the black melanin stage, or

(3) E determines some other condition, such as lower pH, unfavorable to oxidation of the melanogen past the black stage. Gene E may then as well produce the absence of some agent in the

oxidation process (or a lower concentration than ee) as the presence of a "Substance II".

The B gene produces an effect (in the hair) only in the presence of both substances I and II, in which case it produces black pigment in contrast to the chocolate pigment produced by bb. The nature of this difference in action of B and bb is not known, but the present results show that it determines not so much a chemical difference--which should be detectable with the spectrophotometer--but a physical state, such as density or particle size of the pigments involved. Other things equal, the bb animal possesses less pigment than one genetically B, so the action is partially quantitative.

With the background of Wright's hypothesis and the present chemical knowledge of melanin pigments, specific experiments to test the relationship of each gene to the end product (pigment) resulting should be undertaken. The knowledge derived from such studies will be more advantageous in some respects than the correlation of genes with flower pigment chemistry (48) or eye color pigment in Drosophila (10) because of its direct application to physiological, embryological, and genetic processes in mammals. Beadle and Tatum's review (10) is of particular interest because of the possible relationship of the red and black pigments in Drosphila's eyes to mammalian pigments.

SUMMARY

Mammalian integumentary pigments, termed melanins, are chemically similar to the black product produced by tyrosinase acting upon tyrosine and related substrates, and to the red product resulting from the partial oxidation of this black melanin. However, the assumption that melanin pigments are formed in mammals through the action of an anzyme system, either tyrosinase or dopaoxidase, is still unproven. A theory that melanins result from the reaction between protamine and sulfhydrils focuses attention on the latter as biological catalysts in the skin, but cannot be considered established until the compound resulting is shown to have the same spectrophotometric curve as the natural melanins and tyrosinase melanin. Two theories of the nature of color differences in melanin pigmentation have been discussed and it was emphasized that if certain evidence of the spectrophotometric similarity of black and red melanins were upheld, the relation of color to quantitative variations in a single pigment substance would have been demonstrated. Repetition of this work with guinea pig melanins instead of horse melanins, however, has contradicted the previous results and demonstrated that red and black melanins can be spectrophotometrically distinguished. Accordingly the conclusion is drawn that color differences may be due to qualitative, as well as to quantitative, differences. Investigation has further confirmed the theory that red melanin is an oxi-

dation product of black melanin, instead of black melanin being oxidized red melanin. The red intermediate product of the tyrosinase reaction therefore cannot be related to the red melanin occurring in mammalian integuments. Regression coefficients for log (log Io/I) against wave length are proposed as a means of more accurately comparing absorption curves of melanin solutions than has heretofore been done. Their use in further investigations comparing melanins from many different sources and possibly in determining the actual chromogen in mammalian pigment production is suggested. Wright's theories of melanin production as related to guinea pig genes are discussed and certain conclusions regarding the nature of Substance II and the action of the B gene made on the basis of the present experimental results. The goal of workers with mammalian pigmentation should be to establish a complete scheme of the chemical nature and relationships of the pigments involved and to correlate these with the genes responsible.

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